The Utility of 3D Ultramicroscopy for Evaluating Cellular Therapies After Spinal Cord Injury

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Cell therapies have shown promise for repairing the injured spinal cord in experimental models and are now being evaluated in clinical trials for the treatment of human spinal cord injury (SCI). To date, experimental evaluation of implanted cell survival, migration, and integration within the injured central nervous system (CNS) of animals has been technically demanding, requiring tissue sectioning, staining, imaging, and manual reconstruction of 2-dimensional (2D) specimens in 3 dimensions (3D). Not only are these histological procedures laborious and fraught with processing artifacts during manual 3D reconstruction, but they are time-intensive. Herein we describe the utility of 3D ultramicroscopy for assessment of cell therapies after SCI, a new state-of-the-art imaging modality in which whole brain and spinal cord samples are optically sectioned to allow evaluation of intact, macroscopic specimens with microscopic resolution. **Key words:** *cell therapy, Schwann cells, spinal cord injury, 3D ultramicroscopy*

Spinal cord injury (SCI) is a devastating neurological condition, resulting in severe loss of motor, sensory, and autonomic function that, without a significantly effective restorative therapy in the clinic, becomes a major burden on the injured individual, family members, caregivers, and the US health care system. Repairing the injured spinal cord is thus a prominent focus of biomedical research. Experimental studies employing mammalian SCI models have been used to assess the histological and functional efficacy of various pharmacological, molecular, and cellular approaches.[1,2] Our laboratory and others have demonstrated the utility of cell therapies for repairing the injured spinal cord and restoring function after SCI.[3-5] In particular, the implantation of the peripherally derived glial cell, the Schwann cell (SC), can provide significant tissue and cyto-protection,[6-8] support the growth and remyelination of sensory and proprioceptive axons,[6,9] as well as restore function,[6,8-10] an ability analogous to their reparative actions in the injured peripheral nervous system[11] and an ability that can be significantly enhanced through their combination with pharmacological or molecular strategies.[12,13]

To facilitate significant anatomical repair, the implanted cells must survive and integrate within the injured host spinal cord so as to be able to guide axons into, around, and from the lesion. To date, methods for assessing exogenous cell implant survival, migration, integration, and axon growth support have relied upon laborious histological preparation, sectioning, imaging, and computer reconstruction of tissue samples. Not only are such techniques time-consuming and labor-intensive, but they are often prone to the introduction of tissue artifacts, including damage due to mechanical sectioning, tissue folding or loss during staining or mounting, and background immunoreactivity, all of which can severely affect the final interpretation of anatomical outcome measures.

The evaluation of cell therapies for anatomical and functional repair should be conducted in experimental models that best mimic the clinical condition, such as compressive or contusive SCI.

Top Spinal Cord Inj Rehabil 2012;18(1):73–76

www.thomasland.com

doi: 10.1310/sci1801-73

However, in such models, the identification of axon regeneration across the cell implant-lesion is ambiguous; it is impossible to determine the origin and termination of growing axons and thus assess whether they are spared or have sprouted or regenerated in response to implanted cells when such evaluations are performed on tissue sections.[14] Being able to rapidly and reliably assess the survival, migration, integration, and axon growth support of implanted cells in clinically relevant SCI models is thus critically important for understanding how such therapies enhance SCI repair so as to be able to improve or maximize their therapeutic benefit and expeditiously translate them to clinical implementation.

Recently, there has been a revolution in the availability of sophisticated imaging modalities that may provide *in vivo* or *ex vivo* evaluation of whole injured spinal cord tissue and implanted cells in 3D without the need for laborious tissue processing and the associated ambiguity of tracing axons or tracking cells in physically separated tissue sections. These imaging modalities include magnetic resonance imaging (MRI), computerized tomography (CT), diffusion tensor imaging (DTI), 2-photon excitation microscopy, and 3D ultramicroscopy. Such techniques permit the preparation of optical slices of whole tissue, including specifically labeled structures such as exogenous cells, and the 3D computer reconstruction of these macrospecimens at micrometer resolution. Without the need to manually section and stain the specimens, these techniques can greatly reduce consuming time and expensive hands-on labor as wll as the introduction of tissue or staining artifacts.

To perform 3D ultramicroscopy, fixed samples of intact spinal cord are dehydrated in a graded ethanol series and then are cleared in a special chemical clearing solution such that the sample acquires the same refractive index as protein and thereby can be transilluminated with planar sheets of light from an argon-ion laser. Images are recorded from a charge-coupled device (CCD) camera and a microscope positioned above the samples; images are then processed using 3D reconstruction software (Amira 5.2; Visage Imaging, San Diego, USA) under a specific excitation wavelength of 488 nm and emission wavelength of 510 nm.

In the current study, we explored the feasibility of using *ex vivo* 3D ultramicroscopy to assess, within the 3D architecture of the subacutely or chronically injured adult rat spinal cord, the survival, migration, and integration of implanted, fluorescently labeled SCs.

Experimental Methods

Adult female Fisher rats (180-200 g) received a thoracic (T8) moderate spinal cord contusion injury (25.0 mm) using the MASCIS impactor as described previously.[9] Animals were housed in accordance with National Institutes of Health (NIH) guidelines and the Guide for the Care and Use of Animals. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. To allow visualization of the graft, SCs harvested from sciatic nerves were transduced *ex vivo* with lentiviral vectors encoding enhanced green fluorescent protein (EGFP) prior to implantation. At either 1 or 8 weeks (n=5 per time point) post-SCI, $2x10^6$ SCs were injected into the injury epicenter according to previous methods.[9] The animals were perfused 1 month post-SC implantation; the spinal cord tissue specimens were cleared and then imaged using 3D ultramicroscopy.[15] Optical slices were reconstructed in 3D using Amira 5.2 to permit visualization of the spinal cord and SC implant in its entirety for assessment of SC survival, migration, and integration within the host spinal cord. For comparison, a second cohort of similarly treated animals (n=5 per time point) was prepared for standard histological processing and fluorescent microscopy using published procedures.[9]

Results

The use of 3D ultramicroscopy permitted visualization of the SC implants in their entirety within subacutely or chronically contused spinal cords. In both the acute and chronic transplant paradigms, there was evidence of good SC implant survival and almost complete filling of the injury cyst. In agreement with earlier work,[9,10] there was restricted migration of the exogenous EGFP SCs into adjacent host spinal cord tissue. Unlike the laborious methods used to demonstrate SC survival and an absence of cell migration in the earlier work,[9] 3D ultramicroscopy permitted very rapid and unequivocal evaluation of these outcomes in whole spinal cord specimens. Examination of SC implants within the subacutely contused spinal cord revealed large strands of SCs diffusing outwards from the central implant mass in what appeared to be the remnants of the spinal cord vasculature, immediately surrounding the lesion site. This phenomenon was not observed when SCs were implanted chronically. This phenomenon was not readily visible in thin tissue sections, although co-staining of sections from subacutely implanted animals with blood vessel markers, such as tomato lectin, confirmed that implanted SCs were located in these remnant structures. Secondary expansion of the lesion and formation of a well-defined cyst in the chronic injury setting may result in the loss of vasculature remnants, and thus such a phenomenon is not observed when SCs are implanted later post-SCI.

Discussion

In the current studies, we demonstrated for the first time the utility of 3D ultramicroscopy, a novel *ex vivo* imaging technology, to visualize the injured, SC implanted spinal cord in its entirety and permit the rapid evaluation of exogenous cell survival, migration, and integration in acute and chronic injury settings. Analysis of SC implanted spinal cords confirmed our earlier findings of good cell survival and limited outward host cord migration,[9,10] though 3D ultramicroscopy allowed such conclusions to be drawn in a much more rapid and unequivocal fashion. In addition, the use of 3D ultramicroscopy clearly demonstrated a difference in the spatial 3D architecture of the SC implants between acute and chronic transplant conditions, which was not readily apparent following the use of standard histological procedures. Under acute, but not chronic, transplant conditions, EGFP SCs were observed permeating from the central graft mass in outward strands in what appeared to be within the remnants of the vasculature. The implications of such altered implant architecture on SC implant functionality are currently not clear.

In sum, we demonstrated in a clinically relevant contusion model of SCI the macroscopic 3D architecture of exogenous SC implants at microscopic resolution using 3D ultramicroscopy. Future studies will look to further examine implant-host interactions using 3D ultramicroscopy with 2 fluorophores to assess the interaction of implanted SCs with labeled host axon populations or other host elements (eg, the astroglial scar). The ability of 3D ultramicroscopy to rapidly and unequivocally provide information on these anatomical outcome measures will enable more accelerated assessment of cellular (and other) therapeutic approaches for SCI repair and thus ensure faster advancement of such approaches to clinical application for human SCI.

Acknowledgements

This research was funded by Sam Schmidt Paralysis Foundation, American Spinal Injury Association (ASIA) Sell Research Fund, and the Miami Project to Cure Paralysis.

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